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MELATONIN BIOSYNTHESIS IN CHICKEN RETINA

Regulation of Tryptophan Hydroxylase and Arylalkylamine *N*-Acetyltransferase

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1. SUMMARY

Melatonin is synthesized from the amino acid tryptophan (tryptophan → 5-hydroxytryptophan → serotonin → N-acetylserotonin → melatonin). In the chick retina, mRNA levels and activities of two enzymes in this pathway, tryptophan hydroxylase (TPH) and arylalkylamine *N*-acetyltranferase (AA-NAT), display circadian rhythms. The dramatic nocturnal increase in melatonin production in this tissue in part reflects circadian clock-driven increases in mRNA encoding both enzymes. This appears to be translated into increases in both TPH and AA-NAT protein. In the case of AA-NAT, however, this translation is strongly dependent upon environmental lighting. Light acts through post-transcriptional mechanisms to regulate AA-NAT activity; a hypothetical mechanism is proteasomal proteolysis that is otherwise inhibited in the dark by second messengers. Accordingly, melatonin production in the retina, as in the chicken pineal gland, is thought to be regulated by two mechanisms. One is clock-driven changes in TPH and AA-NAT mRNAs, which in turn drive changes in the synthesis of the corresponding encoded proteins. The second is light-induced post-transcriptional degradation of AA-NAT. These mechanisms insure that retinal

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melatonin production follows a precise schedule that reflects daily changes in the environmental lighting.

2. MELATONIN BIOSYNTHESIS IN CHICKEN RETINA

The retinae of many vertebrates have the capacity to synthesize melatonin (44). However, this source of melatonin does not appear to contribute substantially to circulating melatonin, which is pineal derived. Retinal melatonin appears to act locally as a paracrine neuromodulator and regulator of rhythmic retinal physiology (21,23). Retinal melatonin is synthesized primarily in photoreceptor cells (2,10,17,18,42) and its production is regulated by a local, retinal circadian clock (4,10,41).

Melatonin modulates the release of several neurotranmitters in the retina. It inhibits the release of dopamine (6,13) and acetylcholine (29) from amacrine cells. Melatonin stimulates the release of glutamate (15), the photoreceptor neurotransmitter. The release of dopamine and acetylcholine are stimulated by light (e.g., 5,27), while glutamate is released from photoreceptors in darkness (e.g., 34). Thus, melatonin mimics, and may partiially mediate, the effects of darkness on neurotranmitter release. The effect of melatonin on dopamine release appears particularly important, as dopamine is a mediator of circadian changes in visual sensitivity (8,26).

Melatonin promotes dark-adaptive photomechanical movements in photoreceptors and retinal pigment epithelial (RPE) cells (11,30–32,35). Melatonin activates rod photoreceptor disk shedding and phagocytosis by RPE cells (3). Disk shedding and phagocytosis occur in a circadian fashion in most vertebrates, and melatonin may be a neurohumoral link between the retinal circadian clock and the rhythmic turnover of photosensitive outer segment membranes.

Melatonin has effects on photoreceptor survival. Its administration prior to exposure to high intensity light enhances photoreceptor degeneration in albino rats (7,25,43). In contrast, intraocular injection of the melatonin antagonist, luzindole, promotes photoreceptor survival and maintenance of function in light-damaged retinas (36). The mechanisms responsible for these effects are unknown, but may reflect the inhibitory effect of melatonin on dopamine release and loss of a neuroprotective action of dopamine (22). Alternatively, it may reflect direct effects of melatonin on photoreceptor-RPE interactions or a disruption of retinal circadian physiology.

The chicken has become an important experimental model for studying retinal melatonin biosynthesis and actions, because its retina has a relatively high density of melatonin receptors (14,33) and robustly synthesizes melatonin under the influence of a circadian clock (19). Chicken retina contains all of the enzymes of the melatonin biosynthetic pathway and readily converts [^{14}C]trytophan to [^{14}C]melatonin (39). Melatonin levels in chick retina are low during the day, but show a dramatic nocturnal increase in chicks housed under a light-dark cycle or in constant darkness (19). Acute light exposure at night rapidly reduces retinal melatonin levels. This review will focus on the regulation of two enzymes of the melatonin biosynthetic pathway, TPH and AA-NAT, and on their roles in regulating circadian melatonin biosynthesis and the acute inhibitory effect of light in the chick retina.

3. REGULATION OF TPH AND AA-NAT EXPRESSION AND ACTIVITY

3.1. Localization of TPH and AA-NAT mRNAs in Retina

AA-NAT mRNA is localized primarily to the photoreceptor layer of the chick retina, with a lower level of expression in the ganglion cell layer (2); expression was not detected in the inner nuclear layer. TPH mRNA expression is also strong in the photoreceptor layer (12). In addition, TPH mRNA is found in the ganglion cell and inner nuclear layers. The localization of TPH mRNA alone (without AA-NAT mRNA) in the inner nuclear layer corresponds to the previously described serotonin-immunoreactive amacrine cells (28,39). Expression of both mRNAs in photoreceptors is consistent with this cell type as the primary source of retinal melatonin.

Localization of TPH and AA-NAT mRNAs in the ganglion cell layer is unexpected, as serotonin immunoreactivity has not been observed in this cell layer of chick retina. This observation suggests that some ganglion cells may synthesize melatonin or *N*-acetylserotonin. However, the available evidence suggests that melatonin synthesis in ganglion cells is not significant relative to that formed in photoreceptor cells. This is because destruction of most ganglion cells by kainic acid does not decrease melatonin production nor damage photoreceptor cells (39).

3.2. Circadian Rhythms of TPH and AA-NAT mRNAs and Activities

The daily rhythms of TPH and AA-NAT activities are very similar in retinas of chicks exposed to a 12 h light—12 h dark cycle (LD) (Figure 1). Activities of both enzymes are low during the day and show robust increases early in the dark phase of the light-dark cycle. These rhythms of activity appear to be generated, at least in part, by rhythmic expression of TPH and AA-NAT mRNAs (2,12). The daily rhythms of mRNA level and activity are driven by a circadian oscillator and persist in constant light (LL) and constant darkness (DD). The rhythms of mRNA level under constant conditions are very similar for both enzymes, showing peaks in the middle of the subjective dark phase. Thus, expression of the TPH and AA-NAT genes may be regulated by the same clock driven mechanism.

In DD, the rhythms of TPH activity and NAT activity are similar to one another and appear to reflect the expression patterns of their respective mRNAs (2,12,38). In LL, high amplitude TPH activity rhythms occur (Figure 2). Nocturnal activity is only slightly reduced on the first day of LL, but gradually declines over successive days. LL has similar effects on the pattern of TPH mRNA expression (12). In contrast, AA-NAT activity is greatly suppressed (~85%) at night in LL, resulting in a markedly reduced amplitude of the daily rhythm (Figs. 2, 3). The amplitude of the AA-NAT mRNA rhythm is also reduced, but to a much smaller extent (~50%) (Figure 3). This observation indicates that light has a suppressive effect on AA-NAT activity, but not on TPH activity, that may reflect differences in post-transcriptional regulation.

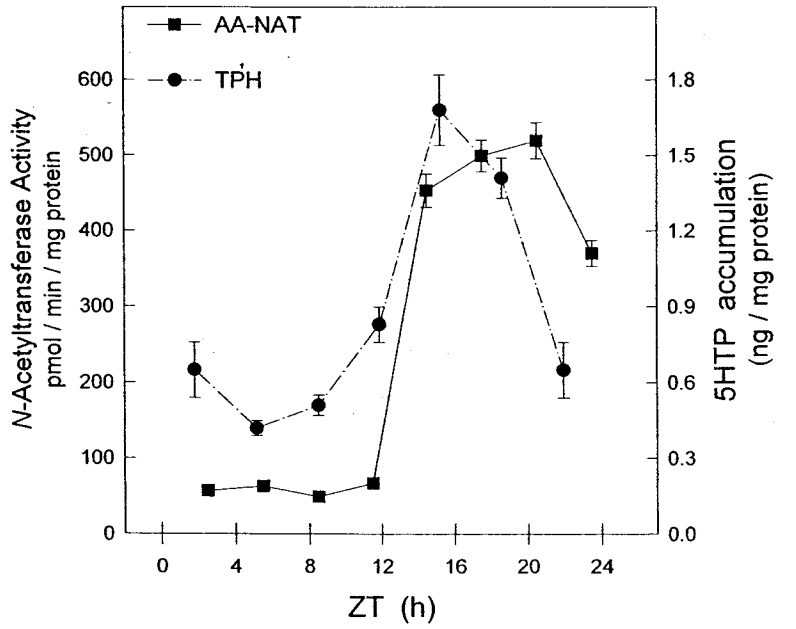

Figure 1. Daily rhythms of TPH and AA-NAT activities in chick retina. Retinas were collected at the zeitgeber times (ZT) indicated during the 12 h light—12 h dark cycle (LD, lights on at ZT 0). TPH activity was estimated from the *in situ* accumulation of 5-hydroxytryptophan (5HTP) 30 min following inhibition of aromatic L-amino acid decarboxylase activity with *m*-hydroxybenzylhydrazine (*m*HBH)(38). AA-NAT activity was measured in retinal homogenates using tryptamine and acetyl coenzyme A as substrates (40). Adapted from Thomas and Iuvone (38) and Iuvone and Alonso-Gomez (23).

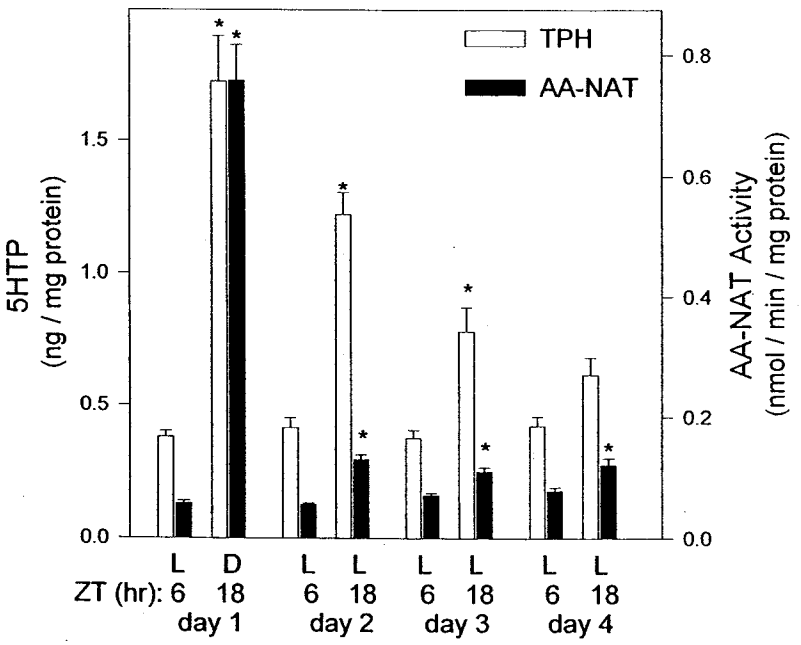

Figure 2. Effect of constant light on the rhythms of TPH and AA-NAT activities. Retinas were dissected 30 min following injection of *m*HBH at midday (ZT 6) and midnight (ZT 18). On day 1, animals were exposed to the entrained LD cycle. Constant light (LL) began on the second day. *$p < 0.01$ *vs* ZT6. Adapted from Thomas and Iuvone (38).

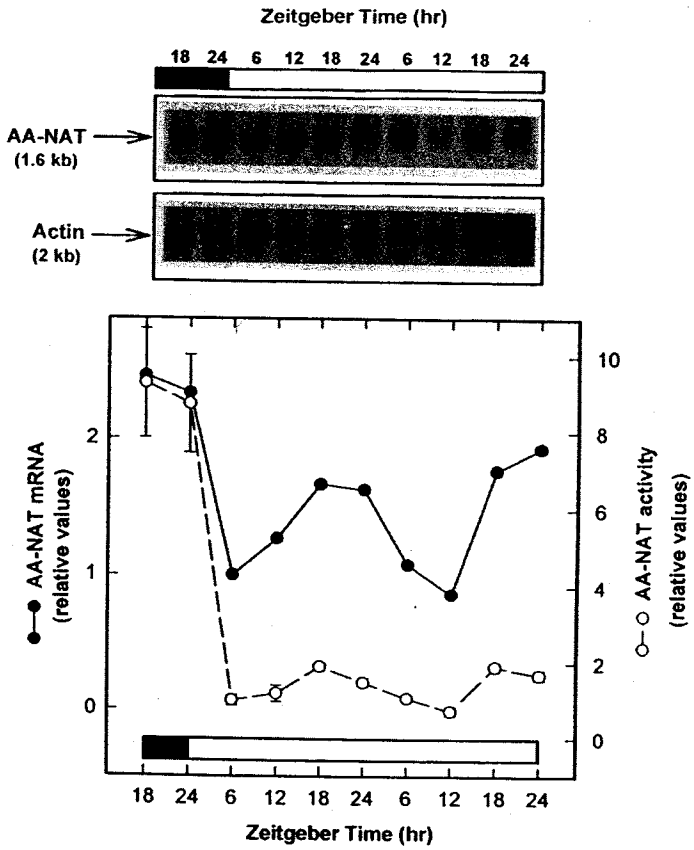

Figure 3. Rhythms of AA-NAT mRNA and activity in LL. Chickens were housed in LD and were then transferred to LL. The open horizontal bar indicates when lights were on and the vertical lines indicate the subjective day/night transitions. Retinas were taken at the indicated times and processed for RNA analysis. The top panels show representative Northern blot analyses of AA-NAT and actin mRNAs. Each lane contains 20μg of total RNA. The bottom panels represent the quantitative analysis of the Northern blots (•–•) and the levels of AA-NAT activity (○–○) in each experimental group. The abundance of the AA-NAT transcript has been normalized to actin mRNA, to correct for variations in loading. All values are expressed relative to the first ZT 6 time point values. AA-NAT activity at ZT 6 was 26 ± 3 pmoles/min/mg protein. Adapted from Bernard et al. (2).

3.3. Effects of Acute Light Exposure on TPH and AA-NAT mRNAs and Enzyme Activities

Light is known to suppress melatonin production by suppressing AANAT activity in both the pineal gland and retina (20,24). In the chicken, it has been found that light exposure during the middle of the dark phase of the LD cycle reduces retinal melatonin levels to daytime values within 1 hour (19). This treatment elicits only small decreases (~30%) of TPH and AA-NAT mRNAs (2,12). Acute light exposure also has little inhibitory effect (~30%) on TPH activity (Figure 4). In contrast, AA-NAT activity is dramatically reduced (~80%) by acute light exposure (Figures 4 and 5). Thus, the effects of acute light exposure on TPH activity appear to reflect mainly changes of mRNA level, while that on AA-NAT activity is elicited by

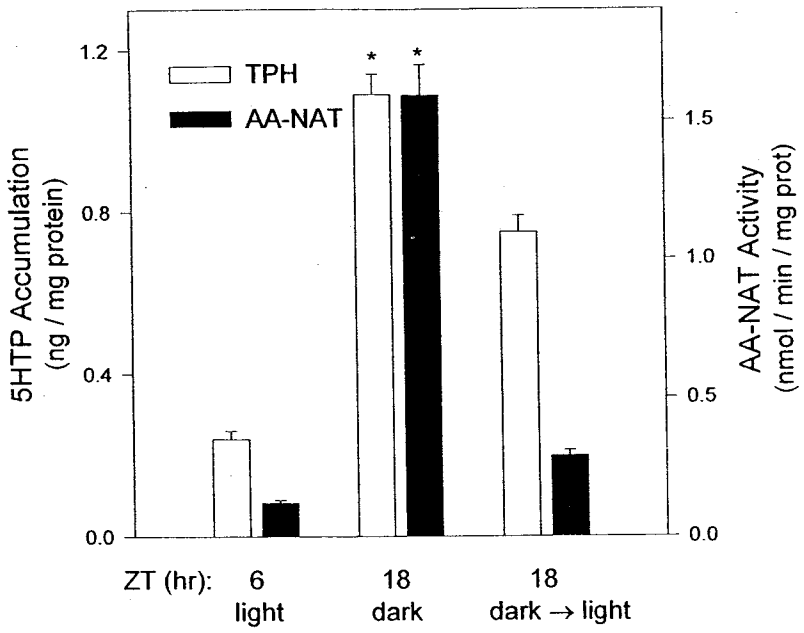

Figure 4. Differential effects of acute light exposure on nocturnal TPH and AA-NAT activities. Animals were exposed to fluorescent room light (2×10^{-4} W/cm^2) during middark phase (ZT 18) for 60 min (Dark → Light). After the initial 30 min of light exposure, they were injected with *m*HBH. Retinas were dissected in light 30 min after injection. Control animals (dark) were treated identically except that they were not exposed to light. Retinas were also obtained from *m*HBH-treated animals during midlight phase (ZT 6). Adapted from Thomas and Iuvone (38).

both decreased mRNA levels and post-transcriptional regulation of enzyme activity. The post-transcriptional regulatory mechanism may involve decreased cyclic AMP levels in the photoreceptor cell resulting in a destabilization of AA-NAT (1). By analogy to the rat pineal gland (16), the destabilization of AA-NAT may result in its proteolysis.

4. ROLES OF TPH AND AA-NAT IN REGULATING MELATONIN BIOSYNTHESIS

The dramatic nocturnal increase of melatonin biosynthesis involves the induction of both TPH and AA-NAT. Similar to the situation in *Xenopus* retina (9), administration of the serotonin precursor, 5-hydroxytryptophan (5HTP), at night greatly increases chick retinal melatonin levels (Figure 6). Administration of tryptophan has no effect. This observation indicates that AA-NAT is not saturated with substrate *in situ*, and that the induction of TPH at night contributes to high rates of melatonin biosynthesis by increasing the supply of serotonin to AA-NAT. In contrast, adminstration of 5HTP during the daytime, when AA-NAT activity is low, elicits a relatively small stimulation of melatonin level (37). Similarly, little increase of melatonin

level is observed following 5HTP administration after acute light exposure at night, which suppresses AA-NAT activity (Fig. 6). Accordingly, it is clear that AA-NAT plays a primary role in regulating melatonin production, and that large changes in serotonin synthesis do not appear sufficient to produce large changes in melatonin production.

The acute inhibitory effect of light exposure on AA-NAT activity appears to result in an accumulation of serotonin sufficient to result in an increase in its oxidation by monoamine oxidase to 5-hydroxyindoleacetic acid (38) (Figure 7). Serotonin continues to be synthesized during light exposure due to high TPH activity, but little of it is *N*-acetylated. The rapid decline in melatonin levels are primarily due to the decrease in AANAT activity, not to a decrease in serotonin, as indicated by the increase in 5-HT and oxidation products that typically results from light exposure.

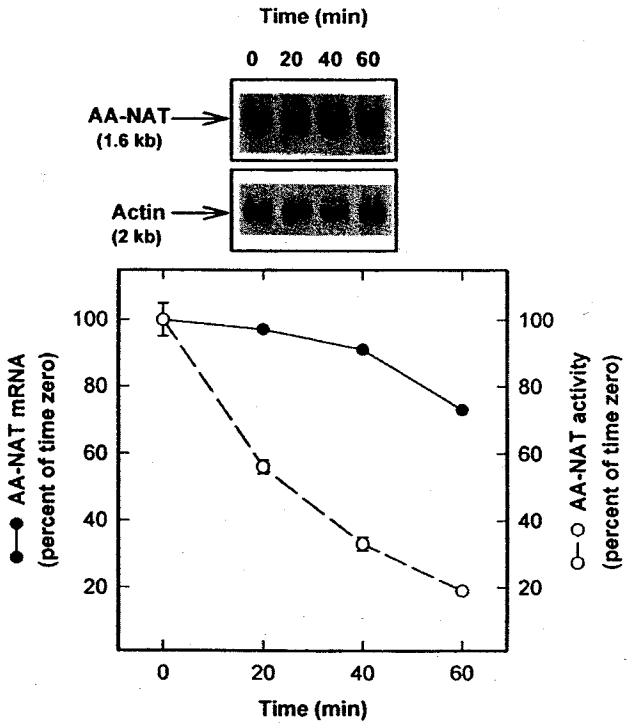

Figure 5. Acute effect of light at night on levels of AA-NAT mRNA and activity. Chickens were housed in LD. Starting at ZT 18 (midnight), the animals were exposed to fluorescent white light for the indicated times. The top panels show representative Northern blot analyses of AA-NAT and actin mRNAs. Each lane contains 20μg of total RNA. The bottom panels represent the quantitative analysis of the Northern blots (•–•) and the levels of AA-NAT activity (○–○) in each experimental group. The abundance of the AA-NAT transcript has been normalized to actin mRNA. All values represent the mean of duplicate determinations (RNA) or the mean ± s.e.m. (activity; n = 8 retinas per group) and are expressed as percent of the t = 0 time point (ZT 18). Individual mRNA values were within 18% of the mean. AA-NAT activity at ZT 18 was 494 ± 67pmoles/min/mg protein. Adapted from Bernard et al. (2).

5. CONCLUSION

These studies are consistent with the hypothesis that induction of both TPH and AA-NAT is essential for the large nocturnal increase of melatonin biosynthesis. The induction of the two enzymes appear to be coordinately regulated by the circadian clock. The activity of TPH is controlled primarily by changes in TPH mRNA level, whereas that of AA-NAT reflects a combination of transcriptional and post-transcriptional regulation. The post-transcriptional regulatory mechanisms appear to be largely responsible for the acute inhibitory effects of light on AA-NAT activity and melatonin levels in the retina.

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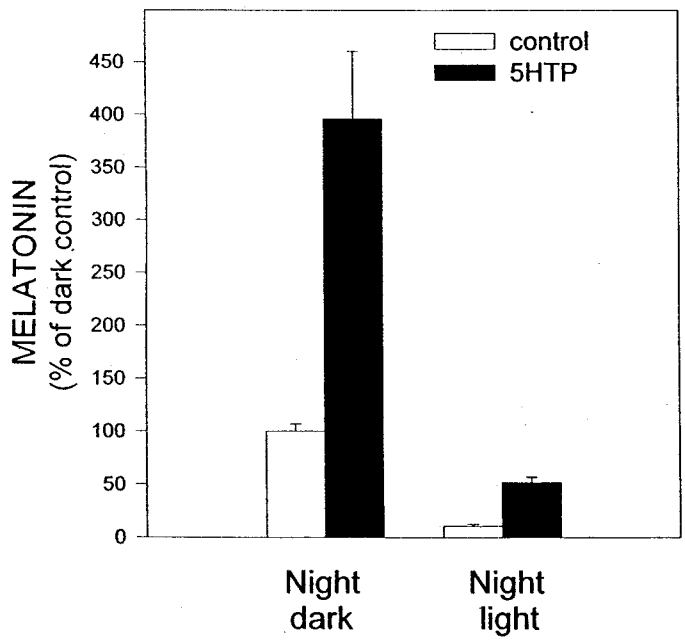

Figure 6. Effect of 5HTP administration on nocturnal melatonin levels in darkness and during acute light exposure. Animals were injected with 5HTP (100 mg/kg ip) during dark phase (ZT15). Retinas were dissected 90 minutes later in darkness (night/dark) or following 90 min exposure to light (night/light). Melatonin content of the retinas was determined by radioimmunoassay. N = 6–8/group. Light exposure significantly reduced nocturnal melatonin level ($p < 0.05$). 5HTP administration significantly increased melatonin levels relative to vehicle controls in both darkness and during acute light exposure, but the increase was much smaller in light-treated retinas. Tryptophan administration (500 mg/kg ip) had no effect on nocturnal melatonin levels. From unpublished data of A.D. Brown, K.B. Thomas, and P.M. Iuvone.

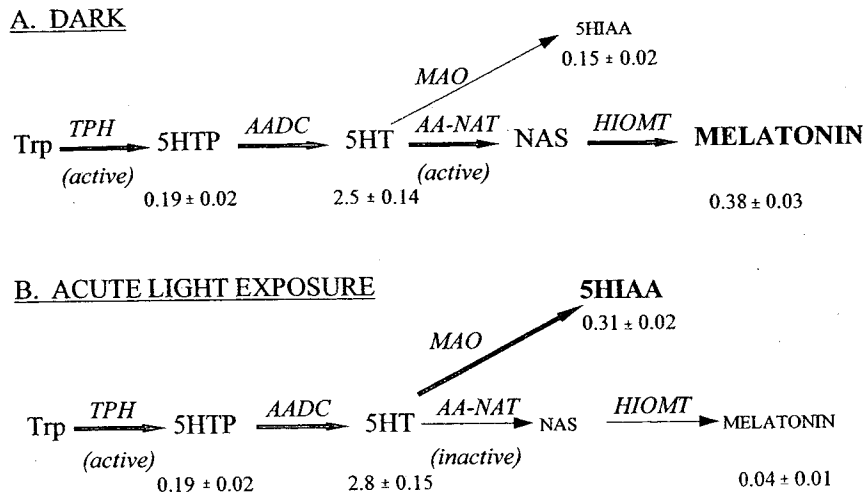

Figure 7. Nocturnal tryptophan metabolism in darkness and following light exposure. A. At night, in darkness, TPH and AA-NAT activities are high, resulting in significant metabolism of trptophan (Trp) to melatonin. B. Following acute light exposure, TPH activity remains high but AA-NAT is inactivated. As a consequence, metabolism in this pathway is shunted from production of melatonin to 5-hydroxyindolacetic acid (5HIAA), a product of serotonin (5HT) oxidation by monoamine oxidase (MAO).Other abbreviations: AADC = aromatic L-amino acid decarboxylase; HIOMT = hydroxyindole *O*-methyltransferase; NAS = *N*-acetylserotonin. Numerical values below 5HTP, 5HT, 5HIAA, and melatonin are steady-state levels expressed in ng/mg protein; values for 5HTP, 5HIAA, and 5HT are from Thomas and Iuvone (38); melatonin values are unpublished data of A.D. Brown and P.M. Iuvone.

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